AD_____

Award Number: W81XWH-12-1-0416

TITLE: Translational Studies of GALGT2 Gene Therapy for Duchenne Muscular Dystrophy

PRINCIPAL INVESTIGATOR: Paul T. Martin, Ph.D.

CONTRACTING ORGANIZATION: The Research Institute at Nationwide Children's Hospital
Columbus, OH 43295

REPORT DATE: October 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual	30September2012-29September2013

4. TITLE AND SUBTITLE
Translational Studies of GALGT2 Gene Therapy for Duchenne Muscular Dystrophy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0416

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Paul T. Martin, PhD
Paul.Martin@nationwidechildrens.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Research Institute at Nationwide Children's Hospital
Columbus, OH 43295

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT- The purpose of this proposal is to perform translational studies in support of developing GALGT2 gene therapy for use in Duchenne Muscular dystrophy patients. In year 1, we have completed the tasks of dosing almost all cohorts of experimental animals to be used in Years 1 and 2 (Wild type or WT, Cmah-/-, mdx and Cmah-/-mdx) via intramuscular injection with the two therapeutic gene therapy vectors to be tested (AAVrh74.MCK.GALGT2 and AAVrh74.MHCK7.GALGT2). We have several major findings from this work, and several experimental problems that need to be resolved. By analyzing functional gene expression in AAV dose response curves, we find that Cmah-/- animals have more expression from rAAVrh74.(MCK or MHCK7).GALGT2 injection at the same dose compared to WT. Likewise, Cmah-/-mdx show higher expression than mdx. These data demonstrate that the human GALGT2 gene may show greater potency in DMD patients than its does in mdx mice due to the altered sialic acid repertoire that exists in humans. Similarly, we find that use of the MHCK7 promoter yielded higher GALGT2 expression at the same AAV dose compared to the MCK promoter. This data suggests that use of the MHCK7 promoter in gene therapy vectors will be more potent in DMD patients. Experimental issues yet to be resolved include understanding the timing of gene overexpression needed to maximize changes in functional measures. 15. Subject Terms. Gene Therapy, Duchenne Muscular Dystrophy,

15. SUBJECT TERMS- Gene Therapy, Duchenne Muscular Dystrophy

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

Introduction	Page 4
Body	Page 4
Key Research Accomplishments	Page 9
Reportable Outcomes	Page 9
Conclusion	Page 10
References	Page 10
Appendices	NA
Supporting Data	NA

Introduction:

Galgt2 overexpression in skeletal myofibers has been demonstrated to protect both wild type and dystrophic muscles from injury and to inhibit the development of muscular dystrophy in three mouse models of human disease, including the mdx mouse model of Duchenne Muscular Dystrophy (DMD)[1-5]. We have developed two gene therapy vectors for use in human DMD clinical trials that allow expression of the human *GALGT2* gene driven by a skeletal muscle-specific promoter (AAV(rh.74)-MCK-*GALGT2*) or cardiac and skeletal muscle-specific promoter (AAV(rh.74)-MHCK7-*GALGT2*). Using these AAV8-like gene therapy vectors, which can cross the vascular barrier, we can effectively deliver *GALGT2* transgene to skeletal muscles via the bloodstream, providing functional correction in mdx mice[1]. In order for such studies to have reference to clinical meaning in human trials, additional dose response studies will be done in this proposal using the mdx mouse and the more severe DMD-like *Cmah*$^{-/-}$mdx mouse, a mouse with a humanized sialoglycome[6]. The objective of the proposed work is to provide pre-clinical data in support of a planned IND application to use *GALGT2* gene therapy to treat Duchenne muscular dystrophy.

Body:

We have made a great deal of progress in year 1 of this study. With the exception of one cohort of Cmah$^{-/-}$ animals, which we had more difficulty in breeding, we have completed Tasks 1-4 for Milestone 1. In addition, we have completed much of Tasks 5-7 of Milestone 2 (for Year 2) in Year 1. For reasons that I will explain, however, we believe additional experiments may be required to fully understand the long-term goal of Milestone 1 and 2. The goal of Milestone 1 was to "Demonstrate Dose Response For Functional Muscle Correction of the EDL in mdx and *Cmah*$^{-/-}$mdx mice 12 weeks after IM delivery of AAV(rh.74)-MCK-*GALGT2*". The goal of milestone 2 was to "Demonstrate Dose Response For Functional Muscle

Correction in mdx and *Cmah*$^{-/-}$mdx mice after IM delivery of AAV(rh.74)-MHCK7-*GALGT2*". We produced both rAAVrh74.MCK.GALGT2 (Task 1, Milestone 1) and rAAVrh74.MHCK7.GALGT2 (Task 5, Milestone 2) in excess of the vg doses needed to complete the proposed studies. We then dosed 11 of 12 cohorts of animals for rAAVrh74.MCK.GALGT2 vectors in Year 1 (Task 2, Milestone 1) and 12 of 12 cohorts for rAAVrh74.MHCK7.GALGT2 (Task 6, Milestone 2). We are still missing one cohort from these experiments, which has now been dosed but is not yet analyzed. After 12 weeks, we then analyzed muscles EDL muscles *ex vivo* for maximal specific force and force drop during eccentric contractions (Task 3, Milestone 1 and Task 7, Milestone 2) and EDL and TA muscles for expression of functional GALGT2, as evidenced by expression of the CT glycan (Task 4, Milestone 1 and Task 8, Milestone 2) and qRT-PCR and Western blot (not yet completed). As will become clear, we likely need to do a few new experiments to obtain the conclusions we were hoping for, but we have made an excellent start on this project. We can make two firm conclusions at this point that we are very excited about.

Figure 1 shows the expression data for the dose response curves of rAAVrh74.MCK.GALGT2 and rAAVrh74.MHCK7.GALGT2 in wild type (WT), Cmah$^{-/-}$, mdx and Cmah$^{-/-}$mdx mice. All mice were injected at roughly 2 weeks of age and analyzed at 12 weeks post-infection. Doses used were $1x10^{10}$vg, $1x10^{11}$vg and $5x10^{11}$vg. Animals dosed at $1x10^{9}$vg showed no significant expression (almost all zeros). We have learned two things of very important significance thusfar from these studies. First, Cmah$^{-/-}$ deletion leads to a significant increase in the percentage of transduced fibers for both rAAVrh74.MCK.GALGT2 gene therapy ($P<0.01$) and for rAAVrh74.MHCK7.GALGT2 gene therapy ($P<0.05$) when compared to wild type muscles. Thus, the human GALGT2 gene may be MORE potent in

muscles where the sialic acid repertoire reflects the repertoire found in humans (Cmah-deleted). This is a very reassuring finding for our planned DMD clinical trial. Second, at the low dose, the percentage of

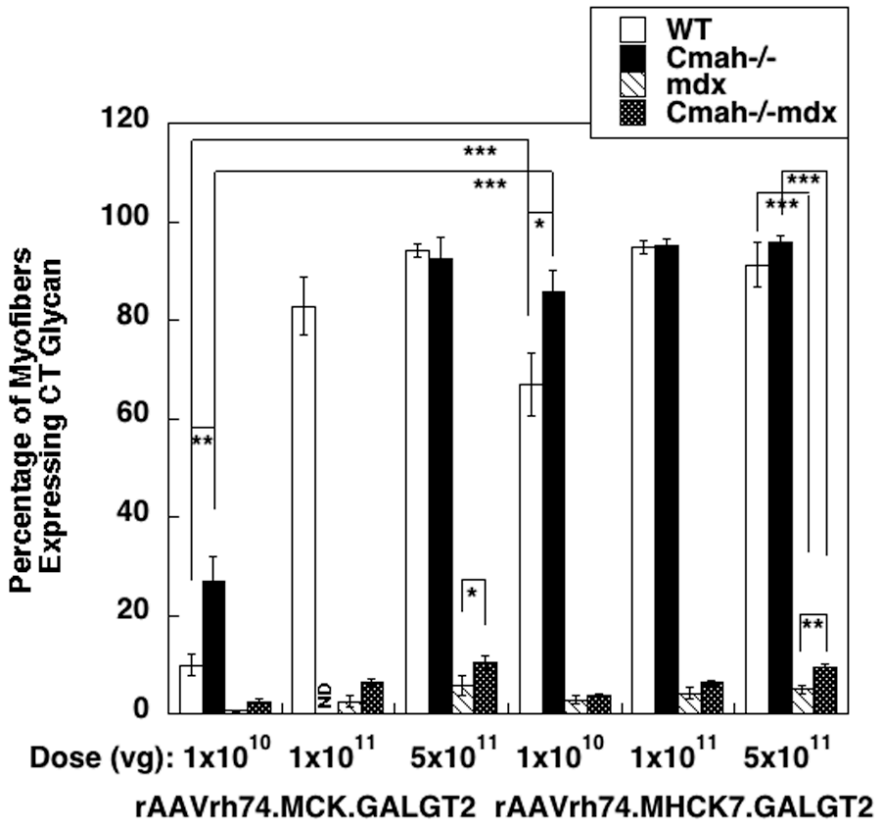

Figure 1. Dose-response curve for rAAVrh74.MCK.GALGT2 or rAAVrh74.MHCK7.GALGT2 treatment. Wild type (WT), Cmah$^{-/-}$, mdx or *Cmah*$^{-/-}$mdx mice were given an intramuscular injection of AAV as indicated in the TA/EDL muscle. Muscles were analyzed for CT glycan overexpression 12 weeks after treatment. Errors are standard errors of the mean for n=10 animals per condition. ND means Not determined. Vg means Vector genomes. Determinations of significance were determined by ANOVA with post-hoc t test.

transduced myofibers is higher in rAAVrh74.MHCK7.GALGT2 treated muscles than for rAAVrh74.MCK.GALGT2 treated muscles ($P<0.001$ for WT and for *Cmah*$^{-/-}$). Obviously,

treated mdx and *Cmah*$^{-/-}$mdx muscles showed very low expression at the same doses compared to wild type and *Cmah*$^{-/-}$ muscles. The most likely explanation for this finding is that it takes 3 weeks for a single-stranded AAV vector to generate maximal gene expression in muscles when a gene is delivered by AAV into a skeletal muscle[7]. We have shown that this is also true for GALGT2[4]. mdx muscles show a profound severe round of muscle damage between 4 and 6 weeks of age, which then tapers off to a slow but progressive myopathy in adult animals[8]. As we injected mice at 2 weeks of age, they would only be reaching maximal gene expression at the peak time of muscle damage. Perhaps this would prevent expression due to elimination of transduced fibers prior to having Galgt2 had time to generate its therapeutic benefit. This would explain the low level of transduction for these two mouse models.

The lack of expression in mdx and *Cmah*$^{-/-}$mdx mucles can be easily corrected in one of two ways. First, we can treat older mdx and *Cmah*$^{-/-}$mdx animals (>2 months) with AAV-GALGT2 vectors, as we have done previously to great effect[1], and measure expression and physiological correction. Second, we can add in a second factor to speed up GALGT2's therapeutic benefit in young animals to prevent the massive tissue loss that occurs at 4-6 weeks in mdx animals. GALGT2 generates its therapeutic benefit via its glycosylation of α dystroglycan (Xu et al, in preparation). mdx muscles show high down-regulation of dystroglycan protein due to this protein's instability in the muscle membrane in the absence of dystrophin[9]. Galgt2 transgenic mdx muscles upregulate dystroglycan such that it reaches wild type levels[2]. So a test of this hypothesis would also be to add an AAV vector to allow expression of human dystroglycan along with GALGT2 in early animals to speed the formation of the CT-glycosylated dystroglycan complex. We have previously shown that overexpression of dystroglycan alone in mdx muscles has no therapeutic benefit, so such an experiment would still

depend on GALGT2 expression[10]. These experiments will be considered in year 2 when analysis of Tasks 1-8 have been fully completed, time permitting. We have already made and produced the gene therapy vector for human dystroglycan.

As one needs greater than 20% of myofibers to see a physical change in muscle strength for GALGT2, we were unsuccessful in demonstrating functional muscle correction in mdx or *Cmah*$^{-/-}$mdx muscles, but we did achieve some functional improvement in specific force (or muscle strength) for certain GALGT2-treated wild type and *Cmah*$^{-/-}$ cohorts. A summary of the high dose data for these findings is given in Figure 2. These data suggest that GALGT2 will work to improve overall muscle strength in mdx and *Cmah*$^{-/-}$mdx muscles once the experimental problems related to expression are solved.

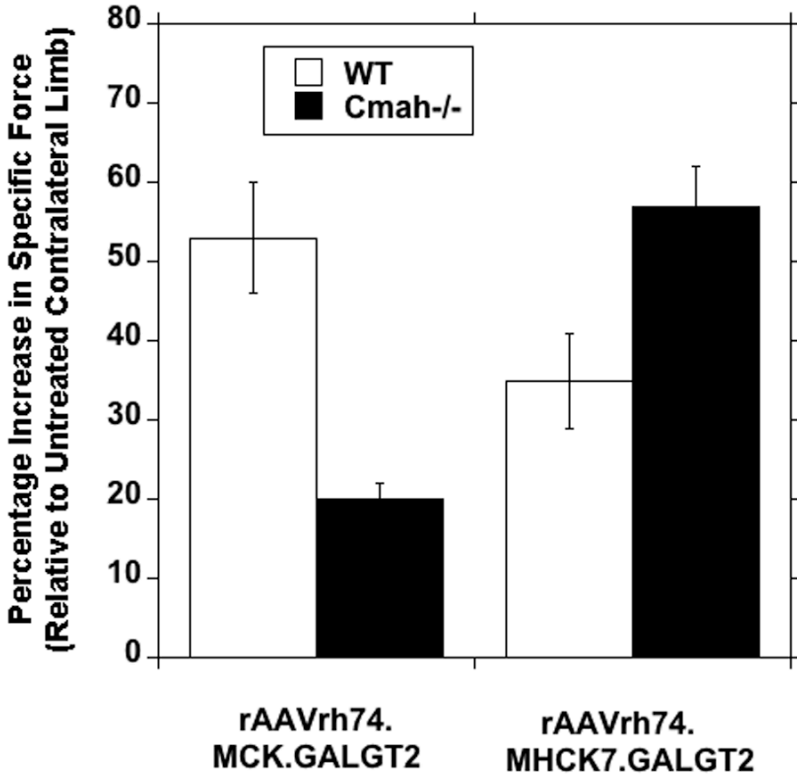

Figure 2. Percentage change in specific force after GALGT2 treatment of wild type (WT) and *Cmah*$^{-/-}$ skeletal muscles. High dose rAAV.MCK.GALGT2 and

rAAV.MHCK7.GALGT2 in some cases led to increased specific force after 12 weeks administration in the extensor digitorum longus muscle measured *ex vivo*. Errors are SEM for n=10 muscles per condition.

Last, we would note that we have made significant progress in our transition plan to move rAAVrh74.MCK.GALGT2 to a clinical trial for DMD. We have filed an application for use of rAAVrh74.MCK.GALGT2 with the Recombinant DNA Advisory Committee (RAC) at the NIH and are in the process of finishing our analysis of GLP toxicology studies for the 6 and 12 week time points. 24-week animals are dose and tissues will be processed in December. This is the last time point in the toxicology studies. We remain on schedule to file an IND with the FDA in January of 2014.

Key Research Accomplishments:

1. Demonstration that human GALGT2 overexpression is more potent in mouse muscles where the Cmah gene is deleted. This deletion better mimics the sialic acid substrates found in human muscle, suggesting that the human GALGT2 gene has more activity when the sialic acid substrates available mimic those found in humans.
2. Demonstration that MHCK7 promoter drives stronger GALGT2 expression than the MCK promoter in mouse skeletal muscle and would therefore be more optimal for use in patients.

Reportable Outcomes:

1. DOD acknowledged in five presentations given by the PI this past year.

April 21-24, 2013 Muscular Dystrophy Association National Scientific Meeting, Washington DC

June 28, 2013 Parent Project Muscular Dystrophy Association Annual Meeting, Baltimore, MD

Sept. 15-20, 2013 EMBO Workshop on Muscle Wasting, Ascona Switzerland

Sept. 30, 2013 Vanderbilt University, Nashville TN

Oct. 1-5, 2013 World Muscle Society Congress (Keynote), Asilomar CA

2. Preliminary data from this award will be used in an R01 grant to the NIH that will be submitted this November 5.

Conclusion: We have made excellent progress in Year 1 of this 3-year award, having met the milestones for Year 1 and completed some, but not all, of the Milestones proposed for Year 2. We have demonstrated that humanizing mouse sialic acids allows the human GALGT2 gene to be more potent. This suggests that the minimally effective dose reported in the mouse for our IND application will be above that we might expect in humans. We have demonstrated that the MHCK7 promoter drives stronger GALGT2 expression than the MCK promoter. This suggests that the use of the MHCK7 promoter may be more efficacious than the currently planned MCK promoter in patient trials for DMD.

References:

1. Martin, P.T.*, et al.* Overexpression of Galgt2 in skeletal muscle prevents injury resulting from eccentric contractions in both mdx and wild-type mice. *Am J Physiol Cell Physiol* **296**, C476-488 (2009).
2. Nguyen, H.H., Jayasinha, V., Xia, B., Hoyte, K. & Martin, P.T. Overexpression of the cytotoxic T cell GalNAc transferase in skeletal muscle inhibits muscular dystrophy in mdx mice. *Proc Natl Acad Sci U S A* **99**, 5616-5621 (2002).
3. Xu, R., Camboni, M. & Martin, P.T. Postnatal overexpression of the CT GalNAc transferase inhibits muscular dystrophy in mdx mice without altering muscle growth or

neuromuscular development: evidence for a utrophin-independent mechanism. *Neuromuscul Disord* **17**, 209-220 (2007).

4. Xu, R., Chandrasekharan, K., Yoon, J.H., Camboni, M. & Martin, P.T. Overexpression of the cytotoxic T cell (CT) carbohydrate inhibits muscular dystrophy in the dyW mouse model of congenital muscular dystrophy 1A. *Am J Pathol* **171**, 181-199 (2007).
5. Xu, R., DeVries, S., Camboni, M. & Martin, P.T. Overexpression of Galgt2 reduces dystrophic pathology in the skeletal muscles of alpha sarcoglycan-deficient mice. *Am J Pathol* **175**, 235-247 (2009).
6. Chandrasekharan, K.*, et al.* A human-specific deletion in mouse Cmah increases disease severity in the mdx model of Duchenne muscular dystrophy. *Sci Transl Med* **2**, 42ra54 (2010).
7. Xiao, X., Li, J. & Samulski, R.J. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* **72**, 2224-2232 (1998).
8. De la Porte, S., Morin, S. & Koenig, J. Characteristics of skeletal muscle in mdx mutant mice. *Int Rev Cytol* **191**, 99-148 (1999).
9. Matsumura, K., Ervasti, J.M., Ohlendieck, K., Kahl, S.D. & Campbell, K.P. Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. *Nature* **360**, 588-591 (1992).
10. Hoyte, K., Jayasinha, V., Xia, B. & Martin, P.T. Transgenic overexpression of dystroglycan does not inhibit muscular dystrophy in mdx mice. *Am J Pathol* **164**, 711-718 (2004).